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(54) Title: METHOD AND DEREGULATED ENZYME FOR THREONINE PRODUCTION

(57) Abstract

Mutagenesis of the gene encoding homoserine dehydrogenase (*hom*) for production of the amino acid threonine is described. The mutation causes an alteration in the carboxy terminus of the enzyme that interferes with end-product inhibition by threonine. The lack of end-product inhibition causes an overproduction of threonine.

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METHOD AND DEREGULATED ENZYME FOR THREONINE PRODUCTION

Background of the Invention

This invention is generally in the area of biochemistry and is specifically directed to production of the amino acid threonine.

Amino acids are often referred to as the building blocks of proteins. Amino acids also serve as sources of nitrogen and sulfur and can be catabolized to provide energy. There are twenty common amino acids all containing at least one carbon atom covalently bonded to a carboxyl group (COOH), an amino group (NH_3), hydrogen (H) and a variable side chain (R).

Amino acids are necessary for the survival of all organisms. Some amino acids are synthesized by the organism while others are provided in the diet. Enzymes transform biomolecules into amino acids, degrade amino acids, and convert amino acids from one type to another. The absence or excess of an amino acid in humans can cause a clinical disorder such as Phenylketonuria, Cystinuria, Fanconi's syndrome or Hartnup disease. Treatment for these disorders currently involves dietary restrictions to reduce intake of the amino acids found in excess and supplementation of the deficient amino acids. The production of large quantities of purified amino acids is essential for scientific research involving amino acid metabolism and treatment of amino acid disorders.

The amino acid threonine has an uncharged polar R group containing a hydroxyl group. The synthesis of threonine proceeds from the substrate aspartate via the branched amino acid biosynthetic pathway as shown in Figure 1. Aspartate is synthesized from oxaloacetate, a product of glucose metabolism through the tricarboxylic acid cycle. Briefly, oxaloacetate is converted to L-aspartate

by a transaminase. L-aspartate is converted to ß-aspartylphosphate by aspartokinase which is dehydrogenated to L-aspartic-ß-semialdehyde which is, in turn, dehydrogenated to L-homoserine by homoserine dehydrogenase encoded by the gene *hom*. Homoserine kinase encoded by the gene *thrB* converts L-homoserine to O-phospho-L-homoserine. Threonine synthase encoded by the gene *thr*C converts O-phospho-L-homoserine to the amino acid L-threonine.

Attempts have been made to produce threonine from bacteria. European Patent Application No. 82104088.8 entitled "Method for Producing L-Threonine by Fermentation" describes high yield producing strains of *Corynebacterium* produced by recombinant techniques. The antimetabolite α-amino-ß-hydroxy-valeric acid is used to screen strains for threonine overproduction. Cells resistant to α-amino-ß-hydroxy-valeric acid toxicity are generally high producers of threonine. Genomic DNA from these resistant strains are inserted into *Corynebacterium* compatible plasmids and used to transform α-amino-ß-hydroxy-valeric acid sensitive strains to produce resistant clones. The gene or genes controlling resistance are not identified or characterized, and threonine production is only achieved with the isolated, resistant strain disclosed. The publication of Eikmanns, et al., *Appl. Microbiol. Biotechnol.*, 34:617:622 (1991) similarly describes a mutant of the *hom* gene designated hom_{fbr}, a homoserine dehydrogenase gene resistant to feedback inhibition by threonine. The hom_{fbr}-*thr*B operon of *C. glutamicum* is expressed in corynebacterial strains for the production of threonine. However, neither the site nor the

region of the mutation causing resistance to feedback inhibition is identified or characterized.

The threonine biosynthetic pathway has been studied extensively in bacteria such as the Gram-positive bacterium *Corynebacterium glutamicum* (*C. glutamicum*), *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*). Although threonine is synthesized via the same reaction path shown in Figure 1 in all three bacteria, the genetic and biochemical organization responsible for the enzymes homoserine dehydrogenase, homoserine kinase, and threonine synthase differ in each organism.

In *Corynebacterium* such as *C. glutamicum*, and *C. flavum*, the homoserine dehydrogenase and homoserine kinase enzymes are encoded by a two-gene operon *hom-thr*B as described by the publications of Follettie *et al.*, Organization and regulation of the Corynebacterium glutamicum *hom-thr*B and *thr*C loci, *Mol. Microbiol.* 2:53-62 (1988) and Peoples *et al.*, Nucleotide sequence and fine structural analysis of the *Corynebacterium glutamicum hom-thrB* operon, *Mol. Microbiol.* 2:63-72 (1988), and U.S. Serial No. 07/062,552 filed June 12, 1987. Transcription of the *hom-thrB* operon is repressed by the amino acid methionine while the activity of homoserine dehydrogenase is allosterically inhibited by the amino acid end product threonine.

The *E. coli* threonine operon (*thr*ABC) encodes four enzyme activities, namely a bifunctional polypeptide, aspartokinase-I-homoserine dehydrogenase-I, a monofunctional homoserine kinase and a threonine synthase. A second bifunctional protein, aspartokinase-II-homoserine dehydrogenase-II, is encoded by the *met*L gene. Expression of the *thr*ABC operon is controlled by threonine-isoleucine

mediated attenuation. Both of the activities encoded by the *thr*A gene, aspartokinase-I-homoserine dehydrogenase-I, are allosterically inhibited by the amino acid threonine.

The *B. subtilis* homoserine dehydrogenase, threonine synthase and homoserine kinase genes are closely linked in the order *hom-thr*C-*thr*B and most likely form an operon. The homoserine dehydrogenase enzyme is repressed by the amino acids threonine and methionine.

In all three bacteria, regulation of threonine synthesis is accomplished by end-product inhibition of the first enzyme in the threonine pathway, the enzyme homoserine dehydrogenase, encoded by the gene *hom or thr*A. The phenomenon of allosteric inhibition of the monofunctional homoserine dehydrogenase enzyme of *C. glutamicum* is characterized in the publication of Follettie *et al.*, *Mol. Microbiol.* 2:53-62 (1988). Threonine inhibits the enzyme with an inhibition rate constant (Ki) of 0.16 mM. Most likely, threonine inhibits the enzymatic activity of homoserine dehydrogenase by binding to a binding site on the enzyme.

Peoples *et al.*, *Mol. Microbiol.* 2:63-72 (1988), have sequenced the *hom* gene of *C. glutamicum* which encodes homoserine dehydrogenase and from this sequence have determined an amino acid sequence encoding a 43,000 dalton polypeptide. The *C. glutamicum* homoserine dehydrogenase exhibits 27% and 31% homology with the *E. coli* and *B. subtilis* homoserine dehydrogenase amino acid sequences respectively.

Attempts have been made to utilize the genes encoding the enzymes involved in threonine biosynthesis to achieve threonine overproduction.

Morinaga *et al.*, *Agric. Biol. Chem.* 51:93-100 (1987) describe transformation of bacterial cells with a plasmid containing both the gene for homoserine kinase from a threonine-producing mutant bacterial strain and the gene for homoserine dehydrogenase. Miwa *et al. Agric. Biol. Chem.* 48:2233-2237 (1984) describe a recombinant *E. coli* strain transformed with a recombinant plasmid containing the threonine operon (*thr*A, *thr*B and *thr*C) of *E. coli*. Nakamori *et al.*, *Chem. Abstracts* 102:216318g (1985) transform *Brevibacterium lactofermentum* with a recombinant plasmid containing the gene for homoserine kinase. Nakamori *et al.*, *Agric. Biol. Chem.* 51:87-91 (1987) transform *Brevibacterium lactofermentum* with a recombinant plasmid containing the gene for homoserine dehydrogenase from *B. lactofermentum* M-15, a threonine and lysine-producing mutant. Takagi *et al.*, *Chem. Abstracts* 106:48643w (1987) transform coryneform bacteria with a recombinant plasmid containing homoserine kinase-encoding genes. The problems with these methods of producing threonine is that the mutations are not characterized, and the resulting plasmids are inherently unstable, resulting in transformed bacteria that are genetically fragile.

What is needed is a stable method of producing threonine that involves a characterized structural mutation. A mutation of the homoserine dehydrogenase gene that prevents end-product inhibition by threonine should result in deregulated threonine biosynthesis.

It is therefore an object of the present invention to provide a method for the overproduction of threonine.

It is a further object of the present invention to provide a method for the production of threonine utilizing a structural mutant.

It is a further object of the present invention to provide a mutation in the homoserine dehydrogenase gene that renders the enzyme insensitive to end-product inhibition.

It is a further object of the present invention to provide a deregulated homoserine dehydrogenase.

Summary of the Invention

A method for the production of threonine and the construction, isolation and cloning of a deregulated homoserine dehydrogenase gene in bacteria is described. A mutation in the gene encoding homoserine dehydrogenase (*hom*) that causes an alteration in the carboxy terminus of the enzyme interferes with end-product inhibition by threonine. The lack of end-product inhibition causes an overproduction of threonine.

Brief Description of the Drawings

Figure 1 (Prior Art) is a schematic diagram of the biosynthesis of threonine from aspartate showing chemical structures and end-product inhibition. Enzyme activities are shown in italic font, substrates and products are shown in Roman font. Allosteric feedback inhibition control is shown with dashed arrows.

Figure 2 is a schematic depiction of sub-cloning strategy and restriction maps. The plasmid pJD1 is an *E. coli* replicon pUC18 containing the 4.1 Kb *Eco*RI R102 genomic DNA encoding replicon exhibiting kanamycin resistance. The plasmid pJD4 is a *C. glutamicum/E. coli* cloning vector pMF33 containing the 3.3 Kb *Sal*I restriction fragment of

pJD1 encoding *hom*^{dr}-*thr*B. The solid black bar represents pSR1 sequences; the shaded bar represents pUC18 sequences; the thin-hatched bar represents Tn903 sequences; the thick-hatched bar represents R102 sequences; and the arrow represents the direction of transcription.

Figure 3 is a graph of relative homoserine dehydrogenase activity measured in crude *C. glutamicum* cell extracts obtained from strains AS019 and its derivative R102 in the presence and absence of L-threonine (L-thr) and DL-α-hydroxy-valerate (AHV) in mMolar. Open square, R102 + L-Thr; dark diamond, AS019 + L-Thr; dark square, R102 + AHV; and open diamond, AS019 + AHV.

Figure 4 is a cartoon of the R102 and AS019 homoserine dehydrogenase and homoserine kinase structural operon showing the location of the preferred single base change associated with the *hom*^{dr} mutation in the carboxy terminus. The predicted amino acid sequence of the deregulated enzyme is compared to the wild type enzyme amino acid sequence in the exploded view.

Figure 5 is a comparison of the *C. glutamicum* deregulated (cghomdr) and the wild type (cghom) homoserine dehydrogenase protein translations with those of the *B. subtilis* homoserine dehydrogenase (bshom) and the *E. coli* aspartokinase-I-homoserine dehydrogenase I (ecthrA) and aspartokinase-II-homoserine dehydrogenase II (ecmet1).

Detailed Description of the Invention

A method for the production of the amino acid threonine and the construction, isolation and cloning of a mutant homoserine dehydrogenase that is insensitive to threonine allosteric inhibition are described. A deregulated mutant homoserine

dehydrogenase gene designated *hom*^{dr} is isolated from the *C. glutamicum* genome and is sequenced. A mutation in the carboxy terminal of the gene results in expression of a truncated homoserine dehydrogenase protein that escapes end-product regulation by threonine, resulting in threonine overproduction.

Methods used in the production and isolation of this mutant can also be used in the production and isolation of other mutants, as described below.

Sequence 1 is the nucleotide sequence and predicted amino acid sequence of *hom*. Sequence 2 is the predicted amino acid sequence of *hom*. Sequence 3 is the nucleotide sequence of the *hom* mutant *hom*^{dr}. Sequence 4 is the predicted amino acid sequence of the *hom* mutant *hom*^{dr}.

Isolation of homoserine dehydrogenase mutant strain

Cells, preferably the cells of bacteria such as *C. glutamicum*, *E. coli* and *B. subtilis* are mutated with a mutagen such as ultraviolet radiation. Most preferably the cells are *C. glutamicum* AS019 cells, a rifampicin resistant prototroph of *C. glutamicum* on deposit with the American Type Culture Collection (ATCC) under ATCC Accession Number 13059. Ultraviolet mutagenesis is preferred over other chemical mutagenic agents because it tends to produce small deletions in the target DNA. Irradiation is preferably performed such that approximately 50% of the irradiated cells are killed.

Mutants producing elevated levels of threonine are screened, preferably by growing the cells on a selective medium such as Minimal Medium *C. glutamicum* (MMCG) plates supplemented with α-hydroxy-valerate, an antimetabolite of threonine.

MMCG is a defined medium well known to those skilled in the art and described in the publication of von der Osten *et al. Biotechnol. Letts.* 11:11-16 (1989). Only cells producing an excess of threonine will survive on a medium supplemented with α-hydroxy-valerate. UV mutagenesis and screening for growth on ever higher levels of α-hydroxy-valerate is continued as necessary.

To enrich for mutations within the threonine pathway, a bioassay is preferably used. An increase in the level of threonine production, a consequence of deregulation of homoserine dehydrogenase, can be approximated by the level of crossfeeding the threonine auxotroph indicator strain AS155. The AS155 indicator strain is described in the publication of Follettie *et al.*, *Mol. Microbiol.* 2:53-62 (1988). Homoserine dehydrogenase activity and sensitivity to L-threonine of isolates that promote growth of the indicator strain after approximately 24 hours is preferably assessed by enzyme assay of crude cell extracts.

A deregulated mutant that is insensitive to threonine, but maintains normal ability to convert L-aspartic-ß-semialdehyde to homoserine was isolated from *C. glutamicum* AS019, and designated R102. This mutant is tolerant to at least 20 mg/ml α-hydroxy-valerate. It encodes a homoserine dehydrogenase activity which is insensitive to threonine, but wild type with respect to its specific activity. The deregulated homoserine dehydrogenase gene (*hom*^{dr}) was cloned from the isolated mutant as described below.

Cloning the deregulated *hom* gene

A plasmid cloning vector, the ampicillin-resistant vector pUC18 was used to clone the gene for the deregulated homoserine dehydrogenase from a

restriction fragment of the mutant genome, a 4.1 Kb *Eco*RI restriction fragment into pUC18. The ligation reaction was used to transform *E. coli* JM83 cells to ampicillin resistance. JM83 cells have the genotype *ara*, D(*lac-pro*AB), *rpo*D, theta80d *lac*ZM15. Recombinant plasmids carrying the 4.1 Kb *Eco*RI chromosomal DNA fragment are identified by *in situ* hybridization with a radiolabelled restriction fragment of the wild type *hom-thr*B operon.

The plasmid designated pJD1 contains the positive clone. The restriction map of pJD1 is shown in Figure 2. The plasmid pJD1 carries the hom^{dr}-*thr*B counter to the adjacent plasmid *lac*Z promoter. In this orientation, the expression of the hom^{dr}-*thr*B operon is controlled by its own promoter located between the *Sma*I and *Hind*III restriction sites.

Expression of hom^{dr} in *C. glutamicum*

The hom^{dr}-*thr*B operon encoded on the plasmid pJD1 was subcloned into the plasmid pMF33 as shown in Figure 2. The plasmid pMF33 is a well known, broad host range, pSR1 replicon, kanamycin resistant *C. glutamicum-E. coli* plasmid. The operon is inserted counter to the *lac* promoter in pMF33. This plasmid is designated pJD4 as shown in Figure 2.

Verified pJD4/AS019EI2 transformants were selected for the presence of the plasmid by growth on kanamycin supplemented mediums. The activity of the homoserine dehydrogenase from crude cell extracts of these cultures was determined in the presence and absence of L-threonine. A host cell encoding wild type homoserine dehydrogenase would be inactivated in the presence of 10 mM L-threonine. The detection of homoserine dehydrogenase activity in the presence of 10 mM L-

threonine indicated that the enzyme was deregulated. Therefore, homoserine dehydrogenase activity in the presence of L-threonine was derived from the cloned *hom*^{dr} gene. The specific activity of the deregulated homoserine dehydrogenase is approximately the same as that reported for the *C. glutamicum* chromosomal *hom* gene (0.0450 μM $ml^{-1}min^{-1}$). The cloned *hom*^{dr} gene product was functional even in the presence of 120 mM L-threonine.

Sequence Analysis of *hom* gene

The complete nucleotide sequence of the *hom*^{dr} gene (Sequence 3) and its promoter region was determined by the Sanger dideoxy method. A comparison of a preferred embodiment of the deregulated gene with the wild type revealed a single nucleotide sequence change at the carboxy terminus of the *hom* gene, a deletion of the guanosine (G) base at coordinate 1964 relative to the upstream *Eco*RI site. The sequence immediately surrounding the site of this hom mutation is shown in Figure 4. The promoter, operator and the *thr*B are conserved. However, the single base pair deletion at 1964 bp disrupts the *hom*^{dr} reading frame at codon 429. This frame shift mutation induces approximately ten amino acid changes and a premature termination, or truncation, i.e., deletion of approximately the last seven amino acid residues of the polypeptide.

It is believed that the single base deletion in the carboxy terminus of the *hom*^{dr} gene radically alters the protein sequence of the carboxyl terminus of the enzyme, changing its conformation in such a way that the interaction of threonine with a binding site is prevented.

It will be understood by those skilled in the art that any mutation in the carboxy terminus

of the *hom*^{dr} gene that prevents end-product inhibition of threonine can be used in a method for the overproduction of threonine as described herein.

The following non-limiting example will demonstrate isolation and characterization of a homoserine dehydrogenase mutant *hom*^{dr} and its gene product, deregulated homoserine dehydrogenase.

Example 1: Isolation of *C. glutamicum* strain R102

Bacterial Strains and Plasmid Constructions

The plasmid maps of the plasmids constructed in this example are shown in Figure 2. Plasmid pMF33, which was used to express *hom*^{dr} is a kanamycin resistant broad host range derivative of the *C. glutamicum* plasmid pSR1 that is capable of replication in both *E. coli* and *C. glutamicum* as described by Archer *et al., Biology of Corynebacterium glutamicum: A molecular approach.* in Genetics and Molecular Biology of Industrial Microorganisms. Washington, American Society for Microbiology (1989). Plasmid pJD1 is a pUC18 derivative containing the 4.1 Kb *Eco*RI R102 chromosomal restriction fragment encoding *hom*^{dr}-*thr*B. Plasmid pJD4 was constructed by ligation of the 3.3 Kb *Sal*I restriction fragment of pJD1 carrying the *hom*^{dr}-*thr*B operon with its promoter into the unique *Sal*I site of pMF33. The *hom*^{dr}-*thr*B operon is orientated counter to the *lac* promoter of pMF33 in pJD4.

Growth Media and Chemicals

E. coli cells were cultured in liquid medium and agar supplemented where necessary with 50 µg/ml kanamycin. *C. glutamicum* was grown in liquid broth medium and Minimal Medium, *C. glutamicum* (MMCG), a defined medium described by von der Osten *et al.*, Biotechnol. Letts. 11:11-16

(1989), supplemented with 50 μg/ml kanamycin when required. The antimetabolite DL-α-hydroxy-valerate was prepared by Carlos Barbas, Texas A&M University (College Station, TX). The substrate for homoserine dehydrogenase assays, aspartate-β-semialdehyde, was synthesized from allylglycine according to the method of Black and Wright, *J. Biol. Chem.* 213:39-50 (1955).

Mutagenesis

Cells were pelleted from a 10 ml exponential phase (OD_{600}=1.0 - 1.2) MMCG culture of *C. glutamicum* AS019 (a rifampicin resistant prototroph of *C. glutamicum* designated ATCC accession number 13059) and washed twice in 10 ml of KCl/phosphate buffer (0.5 M KCl/0.1 M KPO_4, pH = 7.0) in accordance with the method of Sambrok *et al.*, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press (1987). The cells were re-suspended in 1 ml of the same buffer and exposed to 254 nm UV irradiation at a distance of 20 cm for 15 second increments up to 90 seconds. The irradiated cells were serially diluted in phosphate buffer and plated on 10 ml Minimal Medium *C. glutamicum* (MMCG) agar supplemented with between 0.5 and 20 mg/ml α-hydroxy-valerate. Colonies resistant to α-hydroxy-valerate were picked after a 24 hour incubation at 30°C and inoculated into 10 ml MMCG liquid for screening. Subsequent mutagenesis was performed using 60 second exposure which produce 50% killing. Mutations affecting the threonine pathway were identified by the threonine production bioassay described below.

Threonine production bioassay

1 ml of the supernatant from an overnight 10 ml MMCG culture of an α-hydroxy-valerate

tolerant *C. glutamicum* isolate was filter sterilized and added 1:10 to a fresh 10 ml MMCG medium. This was inoculated with the test strain *C. glutamicum* AS155, a threonine auxotroph (a *hom* mutant of AS019). Growth of AS155 was determined by densitometry after a 24 hour incubation at 30°C. The *C. glutamicum* AS019 mutant derivative, R102, was isolated with a tolerance to at least 20 mg/ml α-hydroxy-valerate. The activity of homoserine dehydrogenase in crude cell extracts of *C. glutamicum* R102 and AS019 in the presence of both threonine and α-hydroxy-valerate was measured. Crude cell lysates for homoserine dehydrogenase assays were prepared from 500 ml MMCG *C. glutamicum* AS019 and R102 cultures. The cells were lysed by passage through a French Pressure cell. Homoserine dehydrogenase activity was assayed by determining the initial decrease in the absorbance at 340 nm by NADPH as described by Follettie, *et al., Mol. Microbiol.* 2:53-62 (1988). R102 encoded a homoserine dehydrogenase activity which was insensitive to threonine as shown in Figure 3, but wild type with respect to its specific activity as shown in Table 1 below.

Table 1

Over expression of cloned deregulated homoserine dehydrogenase in *C. glutamicum* AS019E12

Strain/Plasmid	Specific Activity (μM NADPH $ml^{-1}min^{-1}$)	
	+L-threonine (10mM)	-L-threonine
AS019E12/pMF33	0.0	0.045
AS019E12/pJD4	0.507	0.597
AS019	0.0	0.045
R102	0.050	0.0059

R102 therefore encodes a deregulated homoserine dehydrogenase enzyme that is

desensitized to L-threonine and α-hydroxy-valerate but retains the normal catalytic functions for the protein. The deregulated homoserine dehydrogenase gene (*hom*^{dr}) was cloned from the R102 mutant as described below.

Cloning and isolation of *hom*^{dr} from strain R102

The *hom*^{dr} gene was cloned from *C. glutamicum* strain R102 and isolated in *E. coli* using the plasmid cloning vector pUC18, a pMB1 replicon, lacZalpha, ampicillin resistant plasmid. The *hom*^{dr}-*thr*B locus of R102 was cloned as a 4.1 Kb *Eco*RI restriction fragment.

Chromosomal DNA was prepared from *C. glutamicum* R102 and AS019 as follows. A 10 ml liquid broth overnight culture of *C. glutamicum* (R102 or AS019) was inoculated 1:100 into 100 ml of liquid broth and grown with shaking overnight at 30°C. The cells were washed in 20 ml 20 mM Tris-HCl pH 8.0, and resuspended in 10 ml of the same buffer. Protoplasts were made by the stepwise addition of 10 ml 24% polyethylene glycol, 6000 Mr, 2 ml 50 mg/ml lysozyme followed by incubation at 37°C for one hour. The protoplasted cells were harvested and resuspended in 20 ml 100 mM Tris, 10 mM EDTA, pH 8.0, and lysed by the addition of 2 ml 10% sodium dodecyl sulphate and incubated at 55°C until lysis was complete. The cellular debris were removed by centrifugation at 18,000 x g at 4°C. The chromosomal DNA was purified from the cleared lysate by cesium chloride (CsCl) gradient ultracentrifugation.

R102 chromosomal DNA was digested to completion with *Eco*RI. The restriction fragments sized from 3.5 to 5.5 Kb were purified from a 0.6% preparative agarose gel. Bethesda Research Laboratories (Bethesda, MD). These restriction

fragments were ligated with pUC18 which had been linearized and dephosphorylated at the polylinker *Eco*RI site. The ligation reaction was used to transform *E. coli* JM83 cells to ampicillin resistance. JM83 cells have the genotype *ara*, delta(*lac-pro*AB), *rpo*D, theta80d,*lac*ZM15.

Restriction endonucleases and T4 DNA ligase were supplied with the appropriate buffers by Bethesda Research Laboratories (Bethesda, MD). Chromosomal restriction digests of R102 DNA with *Eco*RI were incubated for one hour at 37°C with a ten fold excess of enzyme units. Digestion was terminated by phenol extraction. Plasmid cloning vector DNA was dephosphorylated by incubation with calf intestinal alkaline phosphatase which was purchased from Boehringer-Mannheim Gmbh (Mannheim, Germany). DNA ligations were incubated at 16°C overnight with a ten fold excess of T4 DNA ligase.

Recombinant pUC18 plasmids carrying the 4.1 Kb *Eco*RI chromosomal DNA fragment were identified by *in situ* hybridization with a radiolabelled 1.4 Kb *Kpn*I restriction fragment of the wild type *hom-thr*B operon as follows. Chromosomal DNA isolated from *C. glutamicum* R102 was digested with the following restriction enzymes; *Bam*HI, *Bcl*I, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sph*I, *Sac*I. The restriction fragments were resolved on a 0.8% agarose gel. The nucleic acids were transferred *in situ* to a nitrocellulose filter according to Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 1987. As a probe, nick-translated radioactive DNA was prepared from gel purified 1.4 Kb *Kpn*I generated restriction fragment of plasmid pRA1 (encoding the COOH terminus of the wild type *hom* gene and the NH_2 terminus of the *thr*B gene) also as described by Sambrook *et al*. Radioactively labelled [α^{32}P]-dCTP was supplied by Amersham Corp.

(Arlington Heights, IL). Several thousand recombinant clones were screened, but only one positive clone was identified. This plasmid was designated pJD1. The restriction map for pJD1 is shown in Figure 2.

The *hom*^{dr}-*thr*B operon encoded on plasmid pJD1 was subcloned as a 3.3 Kb *Sal*I restriction fragment encoding the *hom*^{dr}-*thr*B operon and its promoter into the unique *Sal*I site of the broad host range *C. glutamicum-E. coli* plasmid pMF33 as shown in Figure 2. The plasmid pMF33 is a broad host range pSr1 replicon exhibiting kanamycin resistance. The operon was inserted counter to the lac promotor in pMF33. This plasmid was designated pJD4 as shown in Figure 2.

Plasmid pJD4 was introduced by transformation into *E. coli* cells and *C. glutamicum* AS019 to confirm the predicted overexpression of the cloned *hom*^{dr} gene in *C. glutamicum*. *E. coli* cells were transformed with plasmid DNA as described by Cohen, *et al.*, Proc. Natl. Acad. Sci. 69:2110-2115 (1973). *C. glutamicum* AS019 and AS019E12 cells were transformed with plasmid DNA according to the method of Yoshihama *et al.*, J. Bacteriol. 162:591-597 (1985). Verified pJD4/AS019E12 transformants were inoculated into 500 ml MMCG medium supplemented with 50 µg/ml kanamycin to select for the presence of the kanamycin resistant plasmid. After overnight growth at 30°C, the activity of the homoserine dehydrogenase from crude cell extracts of these cultures were determined in the presence and absence of L-threonine. The results of overexpression of the cloned deregulated enzyme in AS019E12 are shown in Table 1 above.

Homoserine dehydrogenase activity in six independent pJD4/AS019E12 isolates was demonstrated in the presence of 10 mM L-threonine, a concentration that would inactivate wild type homoserine dehydrogenase. The specific activity of the deregulated homoserine dehydrogenase (0.0596 μM $ml^{-1}min^{-1}$) was approximately the same as that reported for wild type (0.0450 μM $ml^{-1}min^{-1}$). The level of overproduction of the cloned *hom*^{dr} gene, as determined by specific activity was approximately ten fold higher than production by the chromosomally encoded *hom*^{dr} gene. The cloned *hom*^{dr} gene product was functional in the presence of 120 mM L-threonine.

Plasmid DNA was isolated from *E. coli* using the alkaline lysis technique of Birboim and Doly, Nucl. Acids Res. 7:1513-1514 (1979). Plasmid DNA was isolated from *C. glutamicum* as described by Yoshihama *et al.*, J. Bacteriol. 162:591-597 (1985).

Plasmid DNA sequencing

The complete nucleotide sequence of the *hom*^{dr} gene and its promoter region was determined by the dideoxy-chain terminator method of Sanger *et al.*, *Proc. Natl. Acad. Sci.* 74:5463-5467 (1977) as modified for double stranded DNA sequencing using T7DNA polymerase (Pharmacia, Inc., Piscataway, NJ). This method is described briefly as follows.

A double-stranded DNA sequencing template was prepared by the alkaline lysis technique of Birboim and Doly, *Nucl. Acids Res.* 7:1513-1514 (1979) as described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*. (Cold Spring, Cold Spring Harbor Laboratory Press 1987) from 1.7 ml of an overnight liquid culture of *E. coli* JM83 cells carrying *Exo*III generated unidirectional deletion derivatives of pJD1. The presence of the plasmid

was selected for by the addition of 200 μg/ml ampicillin to the medium. A 5 M sodium acetate solution of nucleic acids was extracted with phenol:chloroform. The nucleic acids were concentrated by ethanol precipitation at -20°C. The pellet was washed in 70% ethanol and dried under vacuum.

DNA annealing and sequencing reactions were as described in the Pharmacia LKB Biotechnology DNA sequence handbook (Pharmacia, Piscataway, NJ). Radioactively labelled [α^{35}S]-dATP was supplied by Amersham Corp. (Arlington Heights, IL).

DNA agarose gels (1.0%, 0.8%, 0.6%) were made with molecular biology grade agarose supplied by Bethesda Research Laboratories (Bethesda, MD). Electrophoresis "E" buffer contained 40 mM tris-acetate, 1 mM EDTA, pH 8.0. DNA was visualized after staining with ethidium bromide (10 μg/ml).

DNA sequence reactions were resolved on 0.6% and 0.8% denaturing polyacrylamide gels (16" x 24") at a constant 55 watts and a current of 5 volts/cm gel length. An electrolyte salt gradient was established by the addition of sodium acetate (0.7 M) to the bottom 1 x TBE buffer, the top buffer was unadulterated 1 x TBE. The gels were prerun for 30 minutes prior to loading. Samples were loaded in 3% ficol, 0.05% bromophenol blue dye. Electrophoresis was continued for 3 hours 30 minutes providing clear separation up to 300 bp from the primer start. Gels were fixed in 12% methanol, 10% glacial acetic acid, 78% water for 20 minutes. The gel matrix was dried under vacuum onto Whatmann 3MM filter paper (Kent, UK) for autoradiography at room temperature overnight.

DNA sequence data was managed using the DNA Inspector IIe program from Textco (West Lebanon,

NH) running on a Macintosh SE/30 computer (Cupertino, CA).

The DNA sequence of the entire *hom*^{dr} gene is shown in sequence 3. The carboxy terminus containing a single base pair deletion of guanosine (G) at coordinate 1964 relative to the upstream *Eco*RI site is shown in Figure 4. The single base pair deletion disrupted the *hom* reading frame at codon 429 and caused a frameshift mutation. The amino acids after codon 429 are different from the wild type. The protein is truncated, having seven amino acids fewer than wild type homoserine dehydrogenase.

Example 2: Homology of the deregulated homoserine dehydrogenase to other homoserine dehydrogenases

To investigate the possible relationships between the region of the homoserine dehydrogenase involved in allosteric inhibition and the other homoserine dehydrogenases, the protein sequences of the deregulated and wild type *C. glutamicum, E. coli* and *B. subtilis* enzymes were compared using the BESTFIT program of the University of Wisconsin Genetic Computer Group software package as shown in Figure 5. The homoserine dehydrogenase sequences were aligned and homology optimized with spaces. The segment of the alignment covering the carboxy termini is presented in Figure 4. A conserved amino acid sequence is centered around residues 439 to 443 in *C. glutamicum* wild type homoserine dehydrogenase. This sequence is deleted from the *hom*^{dr} protein.

Modifications and variations of the present invention will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Archer, John A.C.
Sinskey, Anthony J.
Follettie, Maximillian T.

(ii) TITLE OF INVENTION: Method and Deregulated Enzyme for Threonine Overproduction

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kilpatrick & Cody
(B) STREET: 1100 Peachtree Street, Suite 2800
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: US
(F) ZIP: 30309-4530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: MIT 5424

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404-815-6508
(B) TELEFAX: 404-815-6555

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2340 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corynebacterium glutamicum

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2278..2279
(D) OTHER INFORMATION: /note= "Guanine residue at position 2278 is site of deletion in mutant."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCGACCGCG TGAAGTCGCC CTTTAGGAGA ATTCTGACTA ACTGGAGCCA AAACTTGATC 60

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AGAGGTCTAC TTCAGTTGGT TCACCTTGCA CACAAGCATG AAGTAGTGGG TAGGTCGAGT 180

TGTTAAATGC GGTGTAGAAG GGGAGTAGTT CGCTAGCAAA GGTTAATTTG GAGTCGCTGT 240

ACTGCGGGTT CTCGGGTGGA GTATTCCCGG AGGATTCAAG AAATCTTGAC GCATCTTTGA 300

TGAGGTATGT TTGGAATTCG TCGGCACCTT CCTCGCCGGA GAGGTAGTAG GAGTTCTCGT 360

AATTTGGAAC CCAGATGGCA AATCGTGCGT TTTCGATTGC GTCCAGGACT TCCTCTACGT 420

TGTATCTCGC ACTTGTTGCA GCGGAAGCGA CTCGGTTGCC GATGTCTCCG TATGCAGTGA 480

GCGTGGCGTT TCCGAGGGGA ACTTGATCAG AGGAATACAC CATGGAGCCG ATGTCAGAGG 540

CGACTGCGGG CAGATCCTTT TGAAGCTGTT TCACAATTTC TTTGCCCAGT TCGCGGCGGA 600

TCTGGAACCA CTTTTGCATG CGATCGTCGT CAGAGTGGTT CATGTGAAAA ATACACTCAC 660

CATCTCAATG GTCATGGTGA AGGCCTGTAC TGGCTGCGAC AGCATGGAAC TCAGTGCAAT 720

GGCTGTAAGG CCTGCACCAA CAATGATTGA GCGAAGCTCC AAAATGTCCT CCCCGGGTTG 780

ATATTAGATT TCATAAATAT ACTAAAAATC TTGAGAGTTT TTCCGTTGAA AACTAAAAAG 840

CTGGGAAGGT GAATCGAATT TCGGGGCTTT AAAGCAAAAA TGAACAGCTT GGTCTATAGT 900

GGCTAGGTAC CCTTTTTGTT TTGCACACAT GTAGGGTGGC CGAAACAAAG TAATAGGACA 960

ACAACGCTCG ACCGCGATTA TTTTTGGAGA ATCATGACCT CAGCATCTGC CCCAAGCTTT 1020

AACCCCGGCA AGGGTCCCGG CTCAGCAGTC GGAATTGCCC TTTTAGGATT CGGAACAGTC 1080

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GCTGATGCAG CGGAAGCCGC AAACGTTGAC CTGTACTTCG AGGCTGCTGT TGCAGGCGCA    1440
ATTCCAGTGG TTGGCCCACT GCGTCGCTCC CTGGCTGGCG ATCAGATCCA GTCTGTGATG    1500
GGCATCGTTA ACGGCACCAC CAACTTCATC TTGGACGCCA TGGATTCCAC CGGCGCTGAC    1560
TATGCAGATT CTTTGGCTGA GGCAACTCGT TTGGGTTACG CCGAAGCTGA TCCAACTGCA    1620
GACGTCGAAG GCCATGACGC CGCATCCAAG GCTGCAATTT TGGCATCCAT CGCTTTCCAC    1680
ACCCGTGTTA CCGCGGATGA TGTGTACTGC GAAGGTATCA GCAACATCAG CGCTGCCGAC    1740
ATTGAGGCAG CACAGCAGGC AGGCCACACC ATCAAGTTGT TGGCCATCTG TGAGAACTTC    1800
ACCAACAAGG AAGGAAAGTC GGCTATTTCT GCTCGCGTGC ACCCGACTCT ATTACCTGTG    1860
TCCCACCCAC TGGCGTCGGT AAACAAGTCC TTTAATGCAA TCTTTGTTGA AGCAGAAGCA    1920
GCTGGTCGCC TGATGTTCTA CGGAAACGGT GCAGGTGGCG CGCCAACCGC GTCTGCTGTC    1980
CTTGGCGACG TCGTTGGTGC CGCACGAAAC AAGGTGCACG GTGGCCCTGC TCCAGGTGAG    2040
TCCACCTACG CTAACCTGCC GATCGCTGAT TTCGGTGAGA CCACCACTCG TTACCACCTC    2100
GACATGGATG TGGAAGATCG CGTGGGGGTT TTGGCTGAAT TGGCTAGCCT GTTCTCTGAG    2160
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CAAGGAATCT CCCTGCGTAC AATCCGACAG GAAGAGCGCG ATGATGATGC ACGTCTGATC 2220

GTGGTCACCC ACTCTGCGCT GGAATCTGAT CTTTCCCGCA CCGTTGAACT GCTGAAGGCT 2280

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 445 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corynebacterium glutamicum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Ser Ala Ser Ala Pro Ser Phe Asn Pro Gly Lys Gly Pro Gly
1 5 10 15

Ser Ala Val Gly Ile Ala Leu Leu Gly Phe Gly Thr Val Gly Thr Glu
20 25 30

Val Met Arg Leu Met Thr Glu Tyr Gly Asp Glu Leu Ala His Arg Ile
35 40 45

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Gly Gly Pro Leu Glu Val Arg Gly Ile Ala Val Ser Asp Ile Ser Lys
    50                  55                  60

Pro Arg Glu Gly Val Ala Pro Glu Leu Leu Thr Glu Asp Ala Phe Ala
65                  70                  75                  80

Leu Ile Glu Arg Glu Asp Val Asp Ile Val Val Glu Val Ile Gly Gly
                85                  90                  95

Ile Glu Tyr Pro Arg Glu Val Val Leu Ala Ala Leu Lys Ala Gly Lys
            100                 105                 110

Ser Val Val Thr Ala Asn Lys Ala Leu Val Ala Ala His Ser Ala Glu
        115                 120                 125

Leu Ala Asp Ala Ala Glu Ala Ala Asn Val Asp Leu Tyr Phe Glu Ala
    130                 135                 140

Ala Val Ala Gly Ala Ile Pro Val Val Gly Pro Leu Arg Arg Ser Leu
145                 150                 155                 160

Ala Gly Asp Gln Ile Gln Ser Val Met Gly Ile Val Asn Gly Thr Thr
                165                 170                 175

Asn Phe Ile Leu Asp Ala Met Asp Ser Thr Gly Ala Asp Tyr Ala Asp
            180                 185                 190

Ser Leu Ala Glu Ala Thr Arg Leu Gly Tyr Ala Glu Ala Asp Pro Thr
        195                 200                 205

Ala Asp Val Glu Gly His Asp Ala Ala Ser Lys Ala Ala Ile Leu Ala
    210                 215                 220

Ser Ile Ala Phe His Thr Arg Val Thr Ala Asp Asp Val Tyr Cys Glu
225                 230                 235                 240
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Gly Ile Ser Asn Ile Ser Ala Ala Asp Ile Glu Ala Ala Gln Gln Ala
245 250 255

Gly His Thr Ile Lys Leu Leu Ala Ile Cys Glu Lys Phe Thr Asn Lys
260 265 270

Glu Gly Lys Ser Ala Ile Ser Ala Arg Val His Pro Thr Leu Leu Pro
275 280 285

Val Ser His Pro Leu Ala Ser Val Asn Lys Ser Phe Asn Ala Ile Phe
290 295 300

Val Glu Ala Glu Ala Ala Gly Arg Leu Met Phe Tyr Gly Asn Gly Ala
305 310 315 320

Gly Gly Ala Pro Thr Ala Ser Ala Val Leu Gly Asp Val Val Gly Ala
325 330 335

Ala Arg Asn Lys Val His Gly Gly Arg Ala Pro Gly Glu Ser Thr Tyr
340 345 350

Ala Asn Leu Pro Ile Ala Asp Phe Gly Glu Thr Thr Thr Arg Tyr His
355 360 365

Leu Asp Met Asp Val Glu Asp Arg Val Gly Val Leu Ala Glu Leu Ala
370 375 380

Ser Leu Phe Ser Glu Gln Gly Ile Ser Leu Arg Thr Ile Arg Gln Glu
385 390 395 400

Glu Arg Asp Asp Asp Ala Arg Leu Ile Val Val Thr His Ser Ala Leu
405 410 415

Glu Ser Asp Leu Ser Arg Thr Val Glu Leu Leu Lys Ala Lys Pro Val
420 425 430

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435 440 445

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corynebacterium glutamicum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CACTCGAGAG CTGTGCAGTC TCTTTTTCCT TCAATTCTGC CTGCTCGAGC TCGTAGAAGT 120

AGAGGTCTAC TTCAGTTGGT TCACCTTGCA CACAAGCATG AAGTAGTGGG TAGGTCGAGT 180

TGTTAAATGC GGTGTAGAAG GGGAGTAGTT CGCTAGCAAA GGTTAATTTG GAGTCGCTGT 240

ACTGCGGGTT CTCGGGTGGA GTATTCCCGG AGGATTCAAG AAATCTTGAC GCATCTTTGA 300

TGAGGTATGT TTGGAATTCG TCGGCACCTT CCTCGCCGGA GAGGTAGTAG GAGTTCTCGT 360

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GCGTGGCGTT TCCGAGGGGA ACTTGATCAG AGGAATACAC CATGGAGCCG ATGTCAGAGG     540
CGACTGCGGG CAGATCCTTT TGAAGCTGTT TCACAATTTC TTTGCCCAGT TCGCGGCGGA     600
TCTGGAACCA CTTTTGCATG CGATCGTCGT CAGAGTGGTT CATGTGAAAA ATACACTCAC     660
CATCTCAATG GTCATGGTGA AGGCCTGTAC TGGCTGCGAC AGCATGGAAC TCAGTGCAAT     720
GGCTGTAAGG CCTGCACCAA CAATGATTGA GCGAAGCTCC AAAATGTCCT CCCCGGGTTG     780
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AGCCTGTTGT TAAGGCAATC AACAGTGTGA TCCGCCTCGA AAGGGACTAA TTTTACTGA     2339
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 438 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corynebacterium glutamicum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Ser Ala Ser Ala Pro Ser Phe Asn Pro Gly Lys Gly Pro Gly
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Ser Ala Val Gly Ile Ala Leu Leu Gly Phe Gly Thr Val Gly Thr Glu
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Val Met Arg Leu Met Thr Glu Tyr Gly Asp Glu Leu Ala His Arg Ile
35 40 45

Gly Gly Pro Leu Glu Val Arg Gly Ile Ala Val Ser Asp Ile Ser Lys
50 55 60

Pro Arg Glu Gly Val Ala Pro Glu Leu Leu Thr Glu Asp Ala Phe Ala
65 70 75 80

Leu Ile Glu Arg Glu Asp Val Asp Ile Val Val Glu Val Ile Gly Gly
85 90 95

Ile Glu Tyr Pro Arg Glu Val Val Leu Ala Ala Leu Lys Ala Gly Lys
100 105 110

Ser Val Val Thr Ala Asn Lys Ala Leu Val Ala Ala His Ser Ala Glu
115 120 125

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Ala Gly Asp Gln Ile Gln Ser Val Met Gly Ile Val Asn Gly Thr Thr
                165                 170                 175

Asn Phe Ile Leu Asp Ala Met Asp Ser Thr Gly Ala Asp Tyr Ala Asp
            180                 185                 190

Ser Leu Ala Glu Ala Thr Arg Leu Gly Tyr Ala Glu Ala Asp Pro Thr
        195                 200                 205

Ala Asp Val Glu Gly His Asp Ala Ala Ser Lys Ala Ala Ile Leu Ala
    210                 215                 220

Ser Ile Ala Phe His Thr Arg Val Thr Ala Asp Asp Val Tyr Cys Glu
225                 230                 235                 240

Gly Ile Ser Asn Ile Ser Ala Ala Asp Ile Glu Ala Ala Gln Gln Ala
                245                 250                 255

Gly His Thr Ile Lys Leu Leu Ala Ile Cys Glu Lys Phe Thr Asn Lys
            260                 265                 270

Glu Gly Lys Ser Ala Ile Ser Ala Arg Val His Pro Thr Leu Leu Pro
        275                 280                 285

Val Ser His Pro Leu Ala Ser Val Asn Lys Ser Phe Asn Ala Ile Phe
    290                 295                 300

Val Glu Ala Glu Ala Ala Gly Arg Leu Met Phe Tyr Gly Asn Gly Ala
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Gly Gly Ala Pro Thr Ala Ser Ala Val Leu Gly Asp Val Val Gly Ala
325 330 335

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Ala Asn Leu Pro Ile Ala Asp Phe Gly Glu Thr Thr Thr Arg Tyr His
355 360 365

Leu Asp Met Asp Val Glu Asp Arg Val Gly Val Leu Ala Glu Leu Ala
370 375 380

Ser Leu Phe Ser Glu Gln Gly Ile Ser Leu Arg Thr Ile Arg Gln Glu
385 390 395 400

Glu Arg Asp Asp Asp Ala Arg Leu Ile Val Val Thr His Ser Ala Leu
405 410 415

Glu Ser Asp Leu Ser Arg Thr Val Glu Leu Leu Lys Leu Ser Leu Leu
420 425 430

Leu Arg Gln Ser Thr Val
435

PCT/US92/09325

We claim:

1. A method for the production of threonine comprising constructing a gene encoding an enzymatically active homoserine dehydrogenase not subject to allosteric inhibition.

2. The method of claim 1 further comprising inserting the gene into an expression vector.

3. The method of claim 2 wherein gene is isolated from a genome selected from the group consisting of *Corynebacterium glutamicum*, *Escherichia coli*, and *Bacillus subtilis*.

4. The method of claim 1 wherein the homoserine dehydrogenase gene is mutated at the carboxy terminus.

5. The method of claim 2 further comprising inserting the vector into a host for expression of the gene.

6. The method of claim 4 wherein the mutation is a single base deletion in codon 429 of the *hom* gene of *Corynebacterium glutamicum* resulting in a frameshift.

7. An enzymatically active homoserine dehydrogenase not subject to allosteric inhibition.

8. The homoserine dehydrogenase of claim 7 isolated from bacteria selected from the group consisting of *Corynebacterium glutamicum*, *Escherichia coli*, and *Bacillus subtilis*.

9. The homoserine dehydrogenase of claim 7 wherein the enzyme is altered at the carboxy terminal.

10. The homoserine dehydrogenase of claim 7 wherein the enzyme is a truncated protein.

11. The homoserine dehydrogenase of claim 10 wherein the protein is truncated after codon 438.

12. The homoserine dehydrogenase of claim 7 isolated from *Corynebacterium glutamicum* and having the following nucleotide sequence beginning at codon 423:

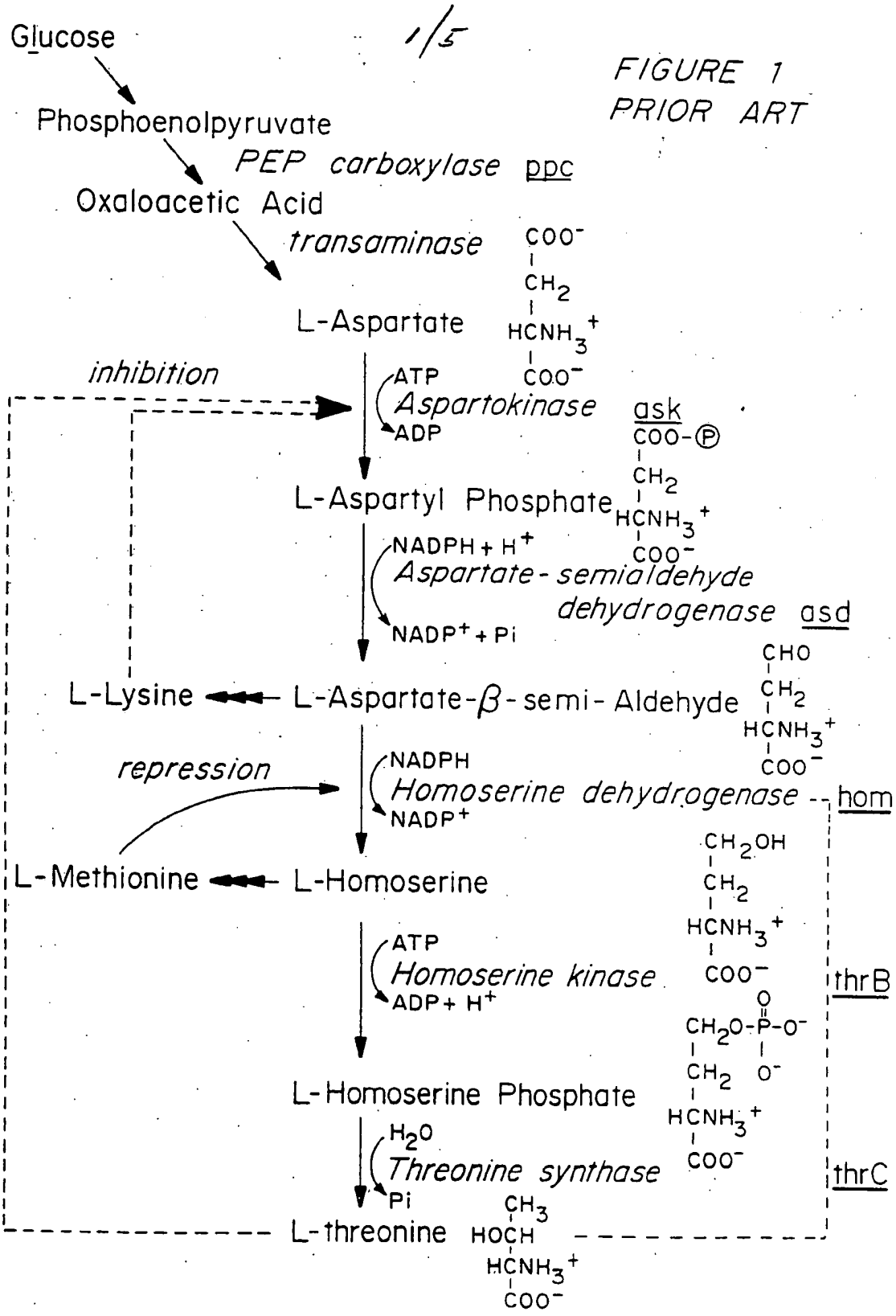
ACC GTT GAA CTG CTG AAG CTA AGC CTG TTG TTA AGG CAA TCA ACA GTG TGA TCC GCC TCG AAA GGG ACT AAT.

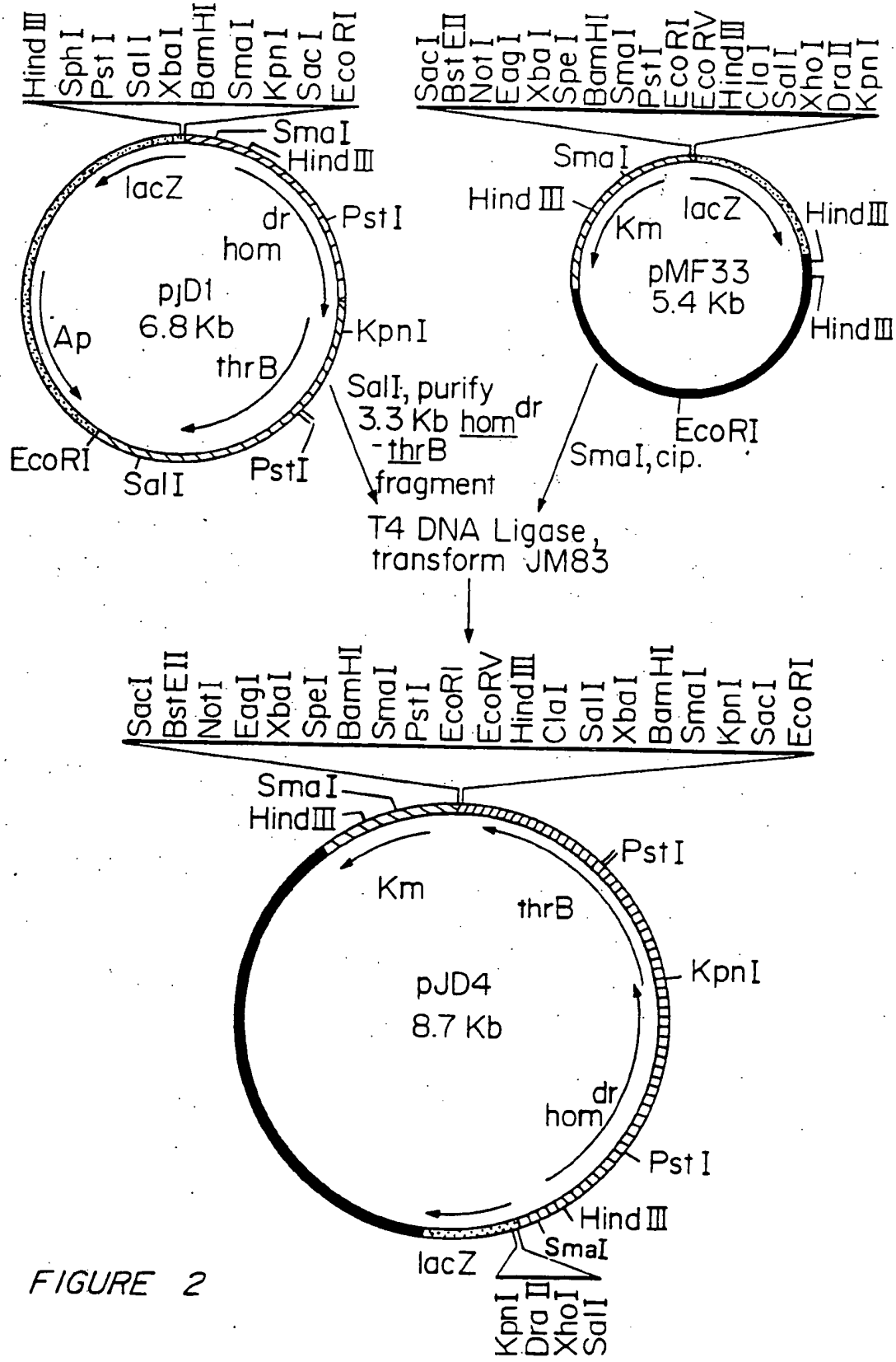
13. The homoserine dehydrogenase of claim 7 isolated from *Corynebacterium glutamicum* and having the following amino acid sequence beginning at codon 423:

Thr Val Glu Leu Lys Leu Ser Leu Leu Leu Arg Gln Ser Thr Val.

1/5

FIGURE 1
PRIOR ART

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Sma I
Hind III
lacZ
dr
hom
Pst I
pjD1
6.8 Kb
Ap
thrB
Kpn I
EcoRI
Sal I
Pst I
Sal I, purify
3.3 Kb hom dr
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fragment
Sma I
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Hind III
pMF33
5.4 Kb
Hind III
EcoRI
Sma I, cip.
T4 DNA Ligase,
transform JM83
Sac I BstEII Not I Eag I Xba I Spe I BamHI Sma I Pst I EcoRI EcoRV Hind III Cla I Sal I Xba I BamHI Sma I Kpn I Sac I EcoRI
Sma I
Hind III
Pst I
Km
thrB
pJD4
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Kpn I
dr
hom
Pst I
Hind III
Sma I
lacZ
Kpn I Dra II Xho I Sal I

FIGURE 2

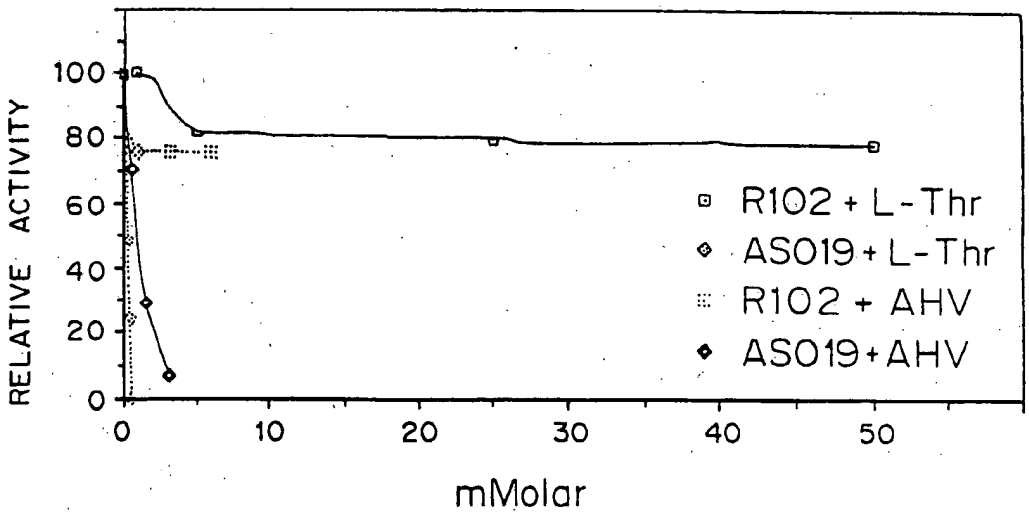
RELATIVE ACTIVITY
100
80
60
40
20
0
0
10
20
30
40
50
mMolar
R102 + L-Thr
AS019 + L-Thr
R102 + AHV
AS019 + AHV

FIGURE 3

FIGURE 4

EcoRI KpnI HindIII
PstI
hom/hom
dr
Phom-thrB
KpnI
PstI
SalI
thrB
wild type
423
Thr.Val.Glu.Leu.Leu.Lys.Ala.Lys.Pro.Val.Val.Lys.
ACC.GTT.GAA.CTG.CTG.AAG.GCT.AAG.CCT.GTT.GTT.AAG.
429
445
Ala.Ile.Asn.Ser.Val.Ile.Arg.Leu.Glu.Arg.Asp.***
GCA.ATC.AAC.AGT.GTG.ATC.CGC.CTC.GAA.AGG.GAC.TAA
deregulated
ACC.GTT.GAA.CTG.CTG.AAG..CTA.AGC.CTG.TTG.TTA.AGG.
Thr.Val.Glu.Leu.Leu.Lys..Leu.Ser.Leu.Leu.Leu.Arg.
423
hom^dr mutation deletion of "G"
CAA.TCA.ACA.GTG.TGA.TCC.GCC.TCG.AAA.GGG.ACT.AAT
Gln.Ser.Thr.Val.***
438

cghomdr	400	E..ERDDDARLIVVTHS.A..LESDL.....SRTVELLKLSLLLRNSTV	438
cghom	400	E..ERDDDARLIVVTHS.A..LESDL.....SRTVELLKAKPVVKAINSVIRLERD	445
bshom	385	K..GHDELAEIVIVTHH.T..SEADF.....SDILQNLNDLEVVQEVKSTYRVEGN	429
ecthra	492	K..NKHIDLRVCGVANSKA..LLTNVHGLNLENWQEELAQAKEPFN.LGRLIRLVKE	543
ecmetl	667	DLSGKDVSRKLVILAREAGYNIEPDQ.....VRVESLVPAHCEGGSIDHFFENGDE	717

FIGURE 5

International application No.
PCT/US92/09325

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 9/04
US CL :435/190

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/190

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS, NTIS, MEDLINE, WORLD PATENT INDEX, BIOTECHNOLOGY ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	See Attached Sheet.	

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 December 1992	Date of mailing of the international search report 25 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer KATHLEEN L. CHOI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09325

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Metabolic Engineering of *Corynebacteria*, issued 1990, "Proceedings of the Sixth International Symposium on Genetics of Industrial Microorganisms", pages 315-325. See entire document.	1-13
X	Journal of Bacteriology, Volume 173, Number 10, issued May 1991, D. J. Reinscheid et al, "Analysis of a *Corynebacterium glutamicum hom* Gene Coding for a Feedback-Resistant Homoserine Dehydrogenase," pages 3228-3230. See entire document.	1-5, 7-9
Y		6,10-13
Y	E.L. Winnacker, "From Genes to Clones: Introduction to Gene Technology", published 1987 by M. Weller (ed.)(Weinheim, New York), pages 451-481. See entire document.	1-13
Y	Molecular Microbiology, Volume 2, Number 1, issued January 1988, O. P. Peoples et al, "Nucleotide Sequence and Fine Structural Analysis of the Corynebacterium gluticum hom-thrB Operon," pages 63-72. See abstract, No. 6976444, Biosis Number: 87036965.	1-13
Y	Genetika, Volume 26, Number 3, issued March 1990, O. Yu Beskrovnaya et al, "Cloning and Structural Analysis in Escherichia coli of Genes of Glutamate Producing Bacteria Involved in Biosynthesis of Aspartic Family of Amino Acids," pages 412-417. See abstract, No. 7693918, Biosis Number: 90061918.	1-13